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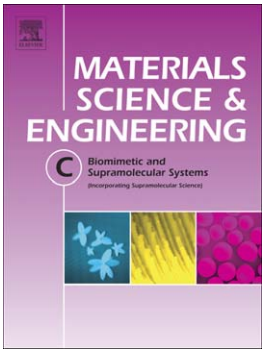

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Jun-zi Wu, ¡!–[INS][David H]–¿David H.¡!–[/INS]–¿ Bremner, He-yu Li, Xiao-zhu Sun, Li-Min Zhu

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Synthesis and Evaluation of Temperature- and Glucose- Sensitive Nanoparticles Based on Phenylboronic Acid and N-Vinylcaprolactam for Insulin Delivery

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ABSTRACT

Poly N-vinylcaprolactam-co-acrylamidophenylboronic acid p(NVCL-co-AAPBA) was prepared from N-vinylcaprolactam (NVCL) and 3-acrylamidophenylboronic acid (AAPBA), using 2,2-azobisisobutyronitrile (AIBN) as initiator. The synthesis and structure of the polymer were examined by Fourier Transform infrared spectroscopy (FT-IR) and ^{1}H-NMR. Dynamic light scattering (DLS), lower critical solution temperature (LCST) and transmission electron microscopy (TEM) were utilized to characterize the nanoparticles, CD spectroscopy was used to determine if there were any changes to the conformation of the insulin, and cell and animal toxicity were also investigated. The prepared nanoparticles were found to be monodisperse submicron particles and were glucose- and temperature- sensitive. In addition, the nanoparticles have good insulin-loading characteristics, do not affect the conformation of the insulin and show low-toxicity to cells and animals. These p(NVCL-co-AAPBA) nanoparticles may have some value for insulin or other hypoglycemic protein delivery.

Keywords: N-Vinylcaprolactam (NVCL), 3-Acrylamidophenylboronic Acid (AAPBA), Nanoparticles (NPs), Glucose-Sensitive, Temperature-Sensitive.

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1. Introduction

Diabetes has become one of the most serious diseases affecting human health [1] and the only effective therapeutic method at present is daily subcutaneous insulin injections which can reduce blood glucose but is unable to intelligently regulate blood glucose levels [2-4]. Moreover, an injection of excess insulin could result in a serious shock response, even leading to death, [5,6] so it is very important to develop smart response systems which release insulin according to blood glucose levels. Recently, work on glucose-sensitivity in physiological conditions has been widely reported [7-10] and includes concanavalin, glucose oxide and phenylboronic acid (PBA) [11], but concanavalin and glucose oxide are both based on proteins and so exhibit certain defects, such as antigenicity, instability and high cost [12-13].

PBA could overcome many of these disadvantages and such derivatives have been proposed as excipients to control the release of drugs from formulations depending on glucose concentration and PBA could provide glucose-sensitive properties involving the B-OH moiety [14]. However, glucose-sensitive polymeric materials based on PBA have several problems. Since the pKa of PBA is much higher than the pH of physiological conditions [15] most of the PBA will be unionized and will therefore have a poor interaction with glucose leading to a weak response capability and, furthermore, PBA has been shown to have some cytotoxicity [16]. Over the years researchers have developed several methods to reduce these drawbacks [17-18] and one of the most useful methods [19] is to modify PBA using polymers that contain carboxyl groups which can coordinate with the boron atoms resulting in a decrease in pKa. Another method [20] is to prepare macromolecular polymers by combing PBA and other materials resulting in polymeric materials which may have a suitable pKa to respond to physiological conditions. Temperature-sensitive materials based on PBA binding have been the most studied [21-23] and thermosensitive polymers that change their solubility below or

above a particular temperature (Lower Critical Solution Temperature or Upper Critical Solution Temperature, LCST or UCST) are often referred to as intelligent hydrogels as they not only can lower the pKa of PBA but also improve the drug loading rate due to their temperature-sensitivity [24].

Recently, poly(N-isopropylacrylamide) p(NIPAAm), a type of temperature sensitive smart gel, has been widely used in drug delivery because it has an ideal LCST close to physiological temperatures. Unmodified p(NIPAAm) gels are swollen and de-swollen below and above 32 ^{o}C, respectively [25]. However, under strongly acidic conditions p(NIPAAm) will yield a toxic organic amine which is undesirable for biomedical applications [26] so alternative temperature-sensitive polymers with low toxicity are required. Poly (N-vinylcaprolactam) p(NVCL) is a temperature-sensitive polymer which has been reported to have a much lower toxicity than p(NIPAAm) [27-28] and consequently there is an increased interest in p(NVCL) for drug delivery systems such as hydrogels, nanoparticles and hybrid particles [29-31]. All of these previous studies have shown that p(NVCL) is very stable to hydrolysis, is biocompatible and is a better choice when used to prepare a potential carrier for biomedical applications.

Bitar et al. [32] produced a temperature- and glucose-sensitive microgel using NVCL and 4-vinylphenylboronic acid (VPBA), but their work did not include drug loading, cumulative drug release and toxicology studies. In addition, VPBA has an obvious disadvantage compared with 3-acrylamidophenylboronic acid (AAPBA) since its water solubility is significantly lower than that of AAPBA, so it may be unsuitable for administration by injection [33]. In the current work, p(NVCL-co-AAPBA) nanoparticles were synthesized, their characteristics were evaluated, loading and insulin release characteristics *in vitro* were studied and their *in vitro* and *in vivo* toxicologies were examined. Furthermore, therapeutic effects were assessed using type 2 diabetes mice and as a result of our research the development of more effective systems for enhanced diabetic control may be envisaged.

2. EXPERIMENTAL DETAILS

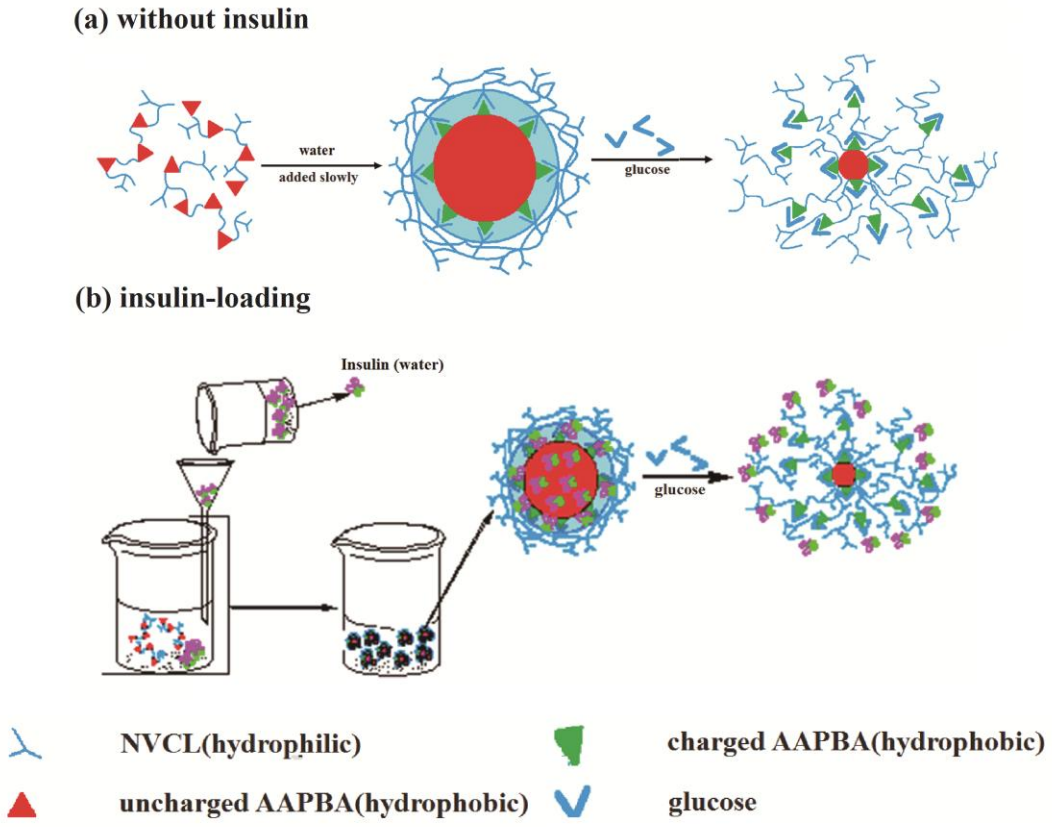
2.1. Materials and Methods

3-Acrylamidophenylboronic acid (AAPBA) was purchased from the Beijing Pure Chem. Co., Ltd. (Beijing, China) and N-vinylcaprolactam (NVCL) was obtained from Hubei Jusheng Co., Ltd. (Wuhan, PR China). Dimethylformamide (DMF) and 2,2-azobisisobutyronitrile (AIBN) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, PR China). Dimethyl sulfoxide (DMSO) was purchased from the China National Medicines Corporation Ltd.

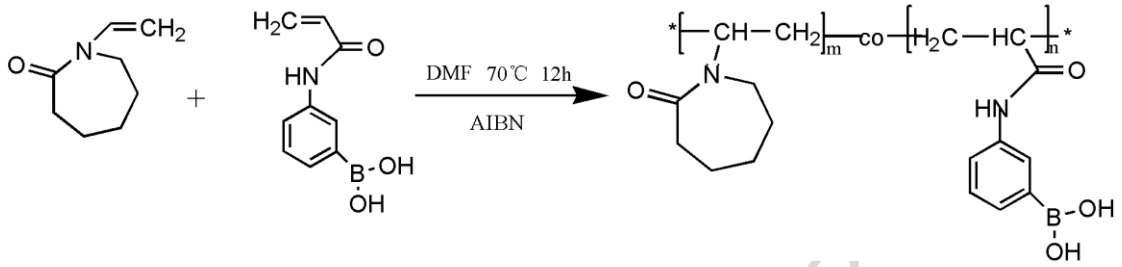
(Beijing, China). All solvents used in this work were of analytical grade and all other reagents were used as received. Water was distilled before use.

2.2. General Synthesis of p(NVCL-co-AAPBA).

p(NVCL-co-AAPBA) was synthesized by a previously reported method [34] using NVCL and AAPBA as monomers, AIBN as the initiator and dimethylformamide as the solvent. The resulting solution was transferred to a sealed test-tube (20 mL) and placed in a pre-heated oil bath and the reaction was carried out under a nitrogen atmosphere for 12 h at 70 ^{o}C. After cooling in ice water for 5 min, the resultant polymer was poured into methanol (1 mL) and the precipitate was washed with diethyl ether, and then isolated by filtration and drying under vacuum. By changing the ratio of AAPBA to NVCL, five distinct polymers were prepared and named as p(NVCL), p(NVCL-co-AAPBA)1, p(NVCL-co-AAPBA)2, p(NVCL-co-AAPBA)3 and p(NVCL-co-AAPBA)4.



Scheme 1. (Graphic abstract) Schematic representation of temperature- and glucose- sensitive p(N-vinylcaprolactam-co-acrylamidophenylboronic acid) p(NVCL-co-AAPBA) nanoparticles.



Scheme 2. The synthesis of p(NVCL-co-AAPBA)

2.3. Characterization of the polymers

Characterization of the polymers using FT-IR, ^{1}H-NMR, GPC and LCST was achieved by methods previously reported by our group [35]. FT-IR spectra were recorded with an FT-IR Prestige-21 spectrophotometer (Shimadzu, Japan) in the 4000–500 cm^{-1} range. ^{1}H-NMR spectra were recorded by dissolving purified samples (3-5 mg) in d6-DMSO (1 mL) using a Bruker DRX 400 MHz spectrometer (Bruker, Rheinstetten, Germany). The molecular weights (Mw and Mn) and molecular weight distributions of the polymers were determined by GPC measurements on a Waters LS system and molecular weight distributions (Mw/Mn) for the sample polymers were calibrated with standard polystyrene samples. The LCST of the polymers were determined using a UV-vis spectrophotometer (Lambda 35, Perkin Elmer, USA) fitted with a thermostatically controlled cuvette at a heating rate of 0.5 ^{o}C/min.

2.4 Preparation of Nanoparticles (NPs)

p(NVCL-co-AAPBA) NPs were prepared according to a previously reported method [36]. A sample of p(NVCL-co-AAPBA) (10 mg) was dissolved in 2 mL of a mixed solvent containing dimethyl sulfoxide (DMSO) and water (v/v, 1:1) and 20 mL of water was then added dropwise to the solution under stirring. The resulting opalescent solution was transferred to a dialysis tube (MWCO 3500) and dialyzed against water for 24 h. The organic solvent was removed by replacing the water every 3 h during the dialyzing process and after freeze-drying the suspension the pure NPs were obtained. The insulin-loaded NPs were prepared by a similar method [36] using 500 µg of insulin dissolved in a sodium acetate solution (1 mL; 0.5%). After dialysis for 24 h, as described above, the suspension of the insulin-loaded NPs was centrifuged at 13000 rpm for 20 min and the amount of free insulin in the supernatant was monitored by the Bradford method using a UV spectrometer (Shimadzu UV-2550) at 595 nm. The insulin Encapsulation Efficiency (EE) and Loading Capacity (LC) were calculated using the following equations:

EE% = (total insulin-free insulin)/total insulin × 100

LC% = (total insulin-free insulin)/NPs weight × 100

2.5 Transmission Electron Microscopy (TEM) and Zeta potential.

The TEM micrographs were obtained by using a transmission electron microscope (JEM-2100, JEOL, Japan). The samples were prepared by casting an aqueous solution of p(NVCL-co-AAPBA) solution (1 mg/mL) onto a copper grid, followed by thin films of Formvar and carbon at 36 ^{o}C. Zeta potential was determined with a submicron particle size analyzer (ZetaPALS/90plus, Brookhaven Instruments Corporation, New York, USA).

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2.6 Reversible pH, glucose and temperature sensitivity of the NPs

p(NVCL-co-AAPBA) NPs were chosen to evaluate sensitivity to changes in pH, glucose concentration and temperature. The pH sensitivity was ascertained by suspending the pure NPs (1 mg) in PBS (1 mL) at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 (obtained by adding NaOH to PBS) for 1 h. Then, the size of the NPs was measured by dynamic light scattering (DLS) at 37 ^{o}C using a Malvern Zetasizer Nano S apparatus equipped with a 4.0 mV laser operating at λ=636 nm. Glucose sensitivity was determined by treating the blank NPs with 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL glucose in PBS (1 mL) for 1 h and the size of the NPs was measured by DLS at 37 ^{o}C. Temperature sensitivity involved suspending the blank NPs (1 mg) in PBS (1 mL, pH 7.4) for 1 h and measuring the size by DLS at 12 ^{o}C, 17 ^{o}C, 22 ^{o}C, 27 ^{o}C, 32 ^{o}C, 37 ^{o}C, 42 ^{o}C and 47 ^{o}C.

2.7 *In vitro* release behavior

In vitro release of different NPs was investigated by incubating insulin-loaded NPs (5 mg) at 37 ^{o}C with 20 mL of pH 7.4 phosphate buffer solution (PBS, 0.1 M) containing different glucose concentrations (0, 1, 2 and 3 mg/mL) under shaking (100 r/min). At predetermined times, some supernatant (1 mL) was withdrawn and fresh buffer solution was added. The amount of free insulin was monitored using UV spectrophotometry with each sample being analyzed in triplicate and the results are reported as mean ±standard deviation (n=3).

2.8 Circular dichroism (CD) measurements

Using the released and standard insulin (2 mg mL^{-1}) in PBS at pH 7.40, CD measurements were performed on a MOS-450 CD spectrophotometer (France Biologic Company, Grenoble, France) at 25 °C with a cell length of 0.1 cm. For the CD spectra the samples were scanned from 195 to 245 nm with a scanning speed of 1 nm per 20s. All CD data were expressed as the mean residue ellipticity.

2.9 Toxicity Assay

2.9.1 Cell viability study and MTT assays.

Cell viability of p(NVCL-co-AAPBA) NPs was evaluated by using human hepatoma SMMC-7721 cells which were cultured in a 1640 medium supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin solution. The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were passaged every 1-2 days by trypsinizing with 0.5%/0.2% (v/v) trypsin–EDTA solution. The cells were seeded into 96-well plates at 10000 cells per well and were incubated for 24 h. Glycopolymer NP suspensions (100 mL) with a range of concentrations from 25 to 500 mg/mL were added to the plates for further incubation. After 24 h, 20 mL of MTT solution (5 mg/mL in PBS buffer) was added to each well and four hours later the medium was removed and the samples in the wells were air dried. DMSO (200 mL) was added to dissolve the crystals which had formed and the optical density of the solution was measured at 570 nm using a microplate reader (Thermo Multiskan MK3, Thermo Scientific Company, Waltham, UK). SMMC-7721 cells, without any treatment, were used as the control.

2.9.2 Acute animal toxicity

A total of 24 Kunming mice (12 male and 12 female) weighing 19-23 g were randomly divided into experimental groups (low, medium and high doses) and the control group (n=6) and were given 100 mg/kg/d, 200 mg/kg/d and 400 mg/kg/d of p(NVCL-co-AAPBA)3 by intraperitoneal injection. By contrast, the control group was given 1 mL/kg/d saline solution by intraperitoneal injection. The eating, activity and death of the mice were observed and after 7 days of injections all mice were sacrificed and their livers, kidneys, spleens, hearts and lungs were collected for the HE staining.

2.9.3 Chronic toxicological experiments

A total of 30 Kunming mice (15 male and 15 female) weighing around 19-23 g were randomly divided into experimental groups (10mg/kg/d, 20 mg/kg/d, 40 mg/kg/d and 80 mg/kg/d doses) and the control group (n=6). Intraperitoneal injections of 10mg/kg/d, 20 mg/kg/d, 40 mg/kg/d and 80 mg/kg/d p(NVCL-co-AAPBA)3 were administered whilst the control group received 1 mL/kg/d saline solution. After 60 days, the mice were sacrificed and their blood was collected and Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin, Hematocrit, Platelet, Serum protein, Serum creatinine, Serum glutathione, Total cholesterol, Glucose, Uric acid, Aspartate aminoTransferase (AST) and Glutamine Transaminase (ALT) were analyzed using an automated Olympus AU5400 biochemistry analyzer (Olympus, Tokyo, Japan).

2.10 *In vivo* hypoglycemic experiments

The diabetes mellitus mice (18) were kindly provided by Kunming Medical University. All of the mice had fasting blood glucose levels of greater than 11.1 mmol/L and were randomly divided into a high fat group (HFG, n=6), an insulin injection group (n=6) and a p(NVCL-co-AAPBA)3 group (n=6). In addition, six healthy mice were selected as a control group (CG). The p(NVCL-co-AAPBA) group received insulin-loaded p(NVCL-co-AAPBA)3 NPs (including 0.4 mg insulin), the insulin injection group received insulin injections (0.16 mg/d) and the HFG group were given distilled water (0.5 mL) injections daily and the CG animals were untreated. Mouse blood was collected from tail veins and blood glucose levels were determined using a Blood Glucose Meter (GT-1640); Jiangxi Jingdou Co., Ltd. Nanchang, PR China) within 60 h of administration. All animal experiment procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1985) and all the experiments were approved by the Animal Care and Use Committee of Donghua University.

3 Results

3.1 FT-IR Spectroscopy.

Fig. 1 shows the FT-IR spectra of NVCL, AAPBA, p(NVCL) and p(NVCL-co-AAPBA)3. NVCL shows C=C str. at 1660 cm^{-1}, C=O str. at 1620 cm^{-1}; CH_2 str. between 2850-3000 cm^{-1} and C=CH str. at 3110 cm^{-1}. AAPBA shows four characteristic absorption bands: C=O str. (1660 cm^{-1}), C=C str. (1620 cm^{-1}), O-B-O (1352 cm^{-1}) and B-O at 1014 cm^{-1}. The benzene ring skeleton is present at 1555 cm^{-1} to 1610 cm^{-1} and the meta-substituted benzene absorptions are at 698 cm^{-1} and 791 cm^{-1}. In p(NVCL) and p(NVCL-co-AAPBA)3, the absorption due to C=C disappear indicating that successful polymerization had occurred. In the FT-IR spectra of p(NVCL-co-AAPBA)3 there exists O-B-O str. at 1340 cm^{-1}, which proves AAPBA had been incorporated into the polymer.

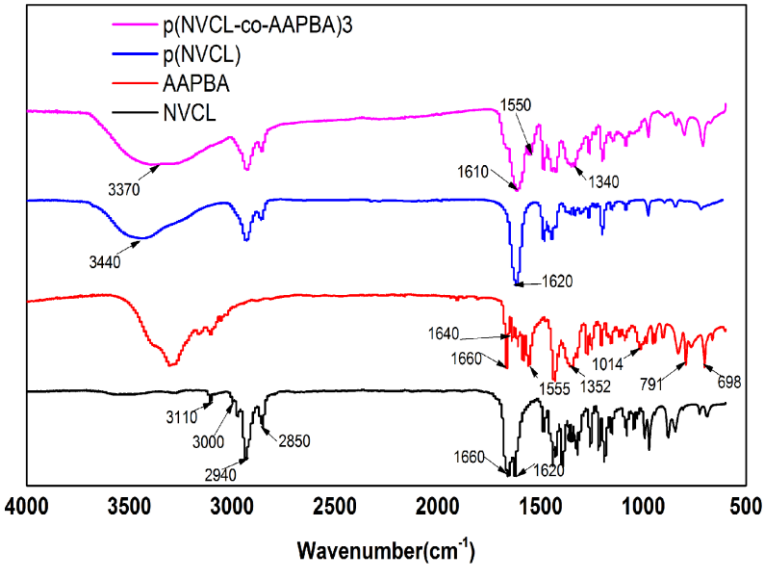

Fig. 1 FT-IR spectra of NVCL, AAPBA, pAAPBA and p(AAPBA-b-NVCL).3

3.2 ^{1}H-NMR Spectroscopy.

Figure 2 shows the ^{1}H-NMR spectra of AAPBA, NVCL, p(NVCL) and p(AAPBA-co-NVCL)3. The spectrum of AAPBA (Figure 2a) shows the following assignments: ^{1}H NMR (DMSO-d6): δ: 10.09 (1H, 4-H), 8.10-7.25 (the H of benzene ring), 6.47 (1H, 2-H), 6.20 (1H, 3-H) and 5.75 (2H, 1-H). NVCL (Figure 2b) (DMSO-d6) δ: 7.23 (1H, 3-H), 5.81 (2H, 2-H), 4.46 (2H, 4-H), 3.35 (2H, 1-H) , 1.62 (2H, 6-H), 2.40 (2H, 5-H) and 1.14 (2H, 7-H). p(NVCL) (DMSO-d6) δ: 4.33 (1H, 4-H), 7.22 (1H, 2-H), 4.33 (2H, 2-H), 1.04 (6H, 1-H) and the p(AAPBA-co-NVCL)3 (DMSO-d6) δ: 9.55 (1H, 1-H), 8.17-7.32 (the H of benzene ring), 7.22 (1H, 2-H), 4.33 (1H, 6-H), 3.19 (2H, 5-H), 2.36 (1H, 4-H) and 1.65 (6H, 3-H). Compared with the spectra of the monomers AAPBA and NVCL, it is obvious that peaks of the ethylene group in p(NVCL) and p(AAPBA-co-NVCL)3 have disappeared and the peaks of these protons can be assigned in the structure of p(AAPBA-co-NVCL)3.

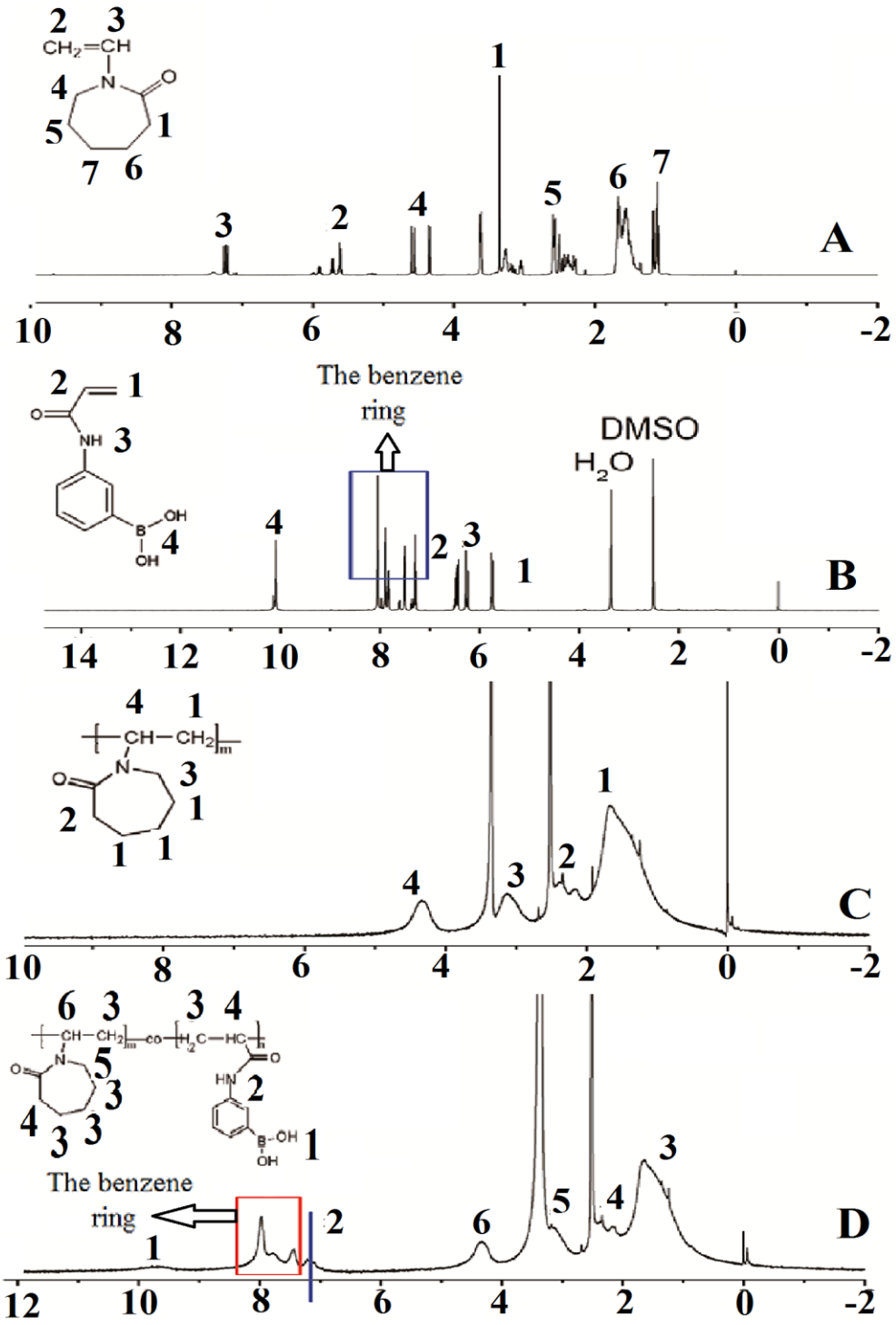

Fig. 2 ^{1}H-NMR spectra of (A) AAPBA; (B) pAAPBA; (C) p(NVCL); and (D) p(AAPBA-b-NVCL)3

3.3 LCST, zeta potential, pH, glucose and temperature sensitivity

The p(NVCL-co-AAPBA) polymers were synthesized via a sequential polymerization method (Scheme 2) and, by changing the ratio of NVCL to AAPBA, five polymers p(NVCL),

p(NVCL-co-AAPBA)1, p(NVCL-co- AAPBA)2, p(NVCL-co-AAPBA)3 and p(NVCL-co-AAPBA)4) were obtained. It was found that with the higher proportion of AAPBA in the p(NVCL-co-AAPBA) co-polymer the LCST, M_w, M_n and the absolute value of zeta potential increased. p(NVCL-co-AAPBA)3 showed a LCST closest to body temperature and zeta potential indicated with increasing the AAPBA in polymers, that the absolute value of the zeta potential is gradually increased (Table 1).

Fig 3 shows the alteration in hydrodynamic diameters with changes in temperature, pH values and glucose concentrations and allowed the best ratio of NVCL to AAPBA to be determined. Fig. 3A shows the changes in NP sizes with the increase of temperature and it can be seen that the diameters of NPs decrease with the increase of temperature, in the order of p(NVCL), p(NVCL-co-AAPBA)1, p(NVCL-co-AAPBA)2, p(NVCL-co-AAPBA)3, p(NVCL-co-AAPBA)4. The p(NVCL-co-AAPBA)4 is the least sensitive to temperature changes (from 188 nm at temperature of 12 ^{o}C to 173 nm at atemperature of 47 ^{o}C) due to the low proportion of NVCL present. p(NVCL) is the most sensitive to temperature changes where the size reduced from 151 nm at temperature of 12 ^{o}C to 108 nm at 47 ^{o}C. The lone pair of electrons in the amide groups (nitrogen atoms) and carbonyl groups (oxygen atoms) are prone to form hydrogen bonds with water resulting in molecules of the polymer being stretched [37]. When the temperature rises, the previously formed hydrogen bonds break, leading to shrinkage of the polymer and hence a decrease in the diameters of the NPs. However, the higher proportion of AAPBA imposes restrictions on the temperature sensitivity of the p(NVCL-co-AAPBA)4.

AAPBA is a Lewis acid, so the OH groups show glucose sensitivity at high pH [38]. Generally, temperature sensitive materials will decrease the pKa of AAPBA but the pH values of human body fluids fluctuate between weakly basic and weakly acidic [39], so it is necessary to examine any pH sensitivity. Thus, the synthesized materials were suspended in PBS solutions with different pHs (from 5 to 9) and the diameters of the NPs were measured at each 0.5 pH value. The results (Fig. 3B) show that the diameters of p(NVCL) and p(NVCL-co-AAPBA)1 show a decrease of 27 nm and 16 nm respectively with the increase in pH and are closely related to the structure of NVCL in the polymer. The N atoms in p(NVCL) are tertiary so the residual lone pair results in the polymer behaving as a base [40]. When the pH value is low, the polymer will be protonated and form a polyelectrolyte resulting in electrostatic repulsion between the NPs leading to stretching of the polymer chains. As the pH increases, the particles are electrically neutral and tend to shrink as they are not protonated. By contrast, the diameters of p(NVCL-co-AAPBA)2, p(NVCL-co-AAPBA)3 and

p(NVCL-co-AAPBA)4 increase by 22nm, 42nm and 48nm, respectively, which mainly results from the incorporation of more PBA into the co-polymer. When the pH value is greater than 6.5, a large amount of the PBA is ionized and the polymers became negatively charged which results in both electrostatic repulsion and hydrogen bonds leading to an increase in the size of the particles.

AAPBA, as the unit of glucose sensitivity, was introduced into the copolymers and the glucose sensitivity of the resulting materials was examined. The normal range of blood glucose is 3.9-6.0 mmol/L (0.1-1.0 mg/mL) compared to that of diabetes patients with 9.0-18.0 mmol/L (1.5-3 mg/mL). If the prepared copolymers responded to a high concentration of glucose, it would have great potential in the application of drug delivery systems with glucose sensitivity. Fig. 3C shows that, as the glucose concentration is raised, the particle sizes of the co-polymers increase significantly. Interestingly, p(NVCL) changes little in size with increase in glucose concentration and that of p(NVCL-co-AAPBA)1 and p(NVCL-co-AAPBA)2 increased only slightly after 1 mg/mL to reach a maximum when the glucose concentration is 2 mg/mL. p(NVCL-co-AAPBA)4 rose from 182 nm at 0.5 mg/mL glucose to 235 nm at 3 mg/mL glucose, however the most impressive change was that of the p(NVCL-co-AAPBA)3 where the size rose from 170 nm at 0.5 mg/mL of glucose to 230 nm at 3 mg/mL. This may be related to the higher proportion of AAPBA in p(NVCL-co-AAPBA). Fig.3D presents the stability of p(NVCL-co-AAPBA)3 and shows that after the addition and removal of glucose the diameters of p(NVCL-co-AAPBA)3 remain stable, indicating the excellent stability of this polymer and hence its potential application as a drug carrier. In addition, the TEM photographs of p(NVCL-co-AAPBA)3 NPs (Fig. 4A) clearly illustrate that the NPs are spherical in shape with good dispersion. Fig. 4B shows that the nanoparticles remained spherical after drug release indicating the stability of prepared NPs.

Table 1. LCST, the molecular weights (Mw and Mn) , Zeta potential and molecular weight distributions and size distribution of self-Assembled Nanomicelles with different ratios

Samples	NVCL/AAPBA	AIBN	Mw	Mn	PDI	LCST	Zeta potential (mV)
P(NVCL)	100/0	2%	66782	54563	1.22	33.0 ^{o}C	-20.8±0.9
P(NVCL-co-AAPB	100/2	2%	6954	56578	1.23	33.5	-24.2±0.7

A)1			3			°C	
P(NVCL-co-AAPBA)2	100/4	2%	71743	58954	1.22	35.5 °C	-33.6±0.6
P(NVCL-co-AAPBA)3	100/8	2%	76432	64389	1.19	37.5 °C	-36.5±0.6
P(NVCL-co-AAPBA)4	100/16	2%	87234	70754	1.23	40.5 °C	-40.6±1.9

Where Mn, Mw and PDI were determined by GPC and LCST by UV-vis spectroscopy.

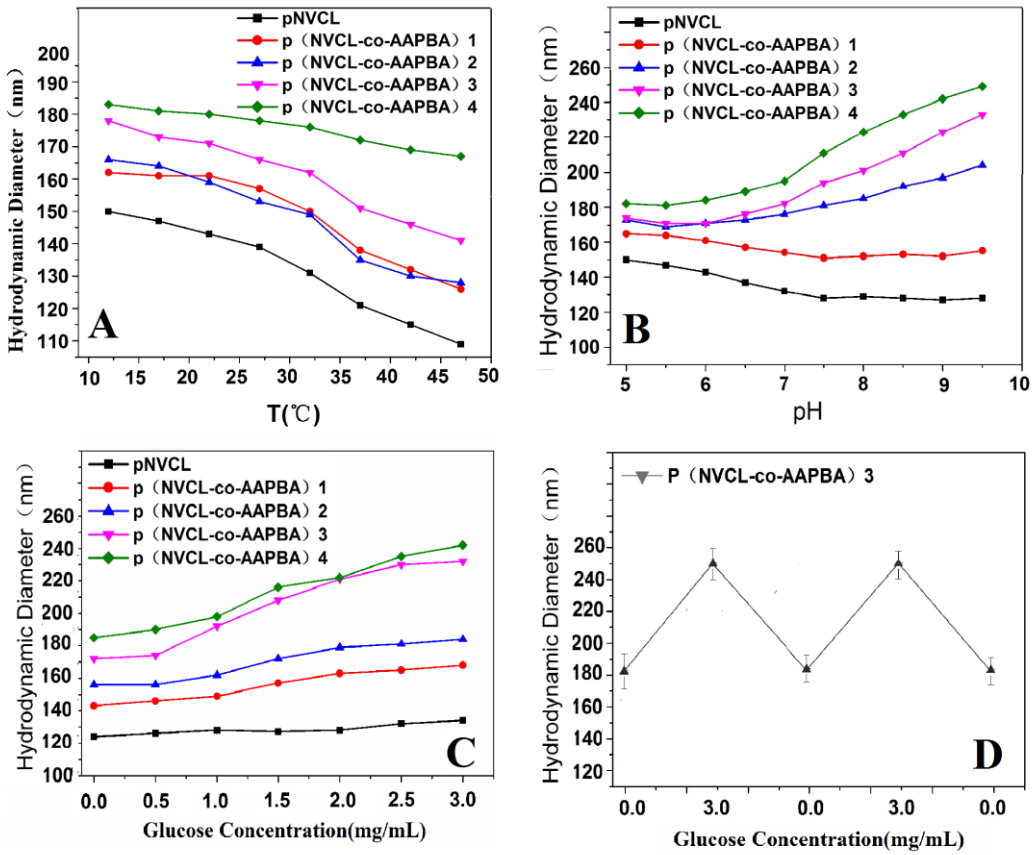

Fig. 3 Hydrodynamic diameters at different: (A) temperatures; (B) pH; (C) glucose concentration; and (D) reversible glucose sensitivity.

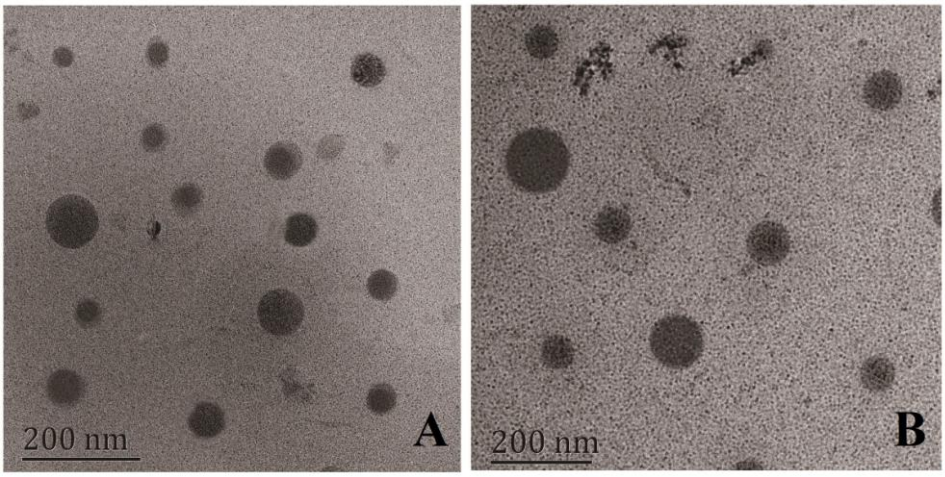

Fig. 4 TEM of p(NVCL-co-AAPBA)3 NPs (A) before and (B) after treatment with 3 mg/mL glucose.

3.4 Insulin loading and release

According to the above results, p(NVCL-co-AAPBA)3 shows the best balance between the temperature and glucose- sensitivity. Therefore, the subsequent experiments, including the drug release behavior, focused on p(NVCL-co-AAPBA)3. The drug loading ratios in this work are higher than the results in some other reports [41-42] probably due to the drug loading method. In the previous work [41], the NIPAM, insulin, SDS, Dex-Ma and AAPBA were mixed, then purged with nitrogen, and the reaction was allowed to proceed for 8 h in 70 ^{o}C, to directly generate the insulin-loaded P(NIPAM–Dex–PBA) nanogels. Other differences in material properties may be due to the method of insulin-loading; the use of D-gluconamidoethyl methacrylate as the copolymer; and the fact that they used a DMSO:H_2O ratio of 4:1 whilst the current work used a ratio of 1:1 [42]. The drugs were loaded at low temperature when the particles began to swell but as the temperature was increased, the particles shrank, which ensured the embedding of the drugs. At low temperature the low temperature sensitive polymer stretch, so they have more change to absorb the drugs [43]. When the temperature rises, the drug will be shrink of, ensure the embedding of the Insulin. When different concentrations of Insulin were studied and 10 mg of p(NVCL-co-AAPBA)3 was used as the carrier the results (Table 2) show that with the higher concentrations of insulin, LC(%) increased but EF(%) decreased. The drug release behaviors of the five samples were tested at a pH of 7.4 and a temperature of 37 ^{o}C and it was found when the loading of insulin to polymer was 1.5 [termed (Insulin-loading)4 in Fig. 5A] the drug release amount was optimum as this loading shows the best balance between the amount

of the drug, encapsulation efficiency and cumulative release. Thus (Insulin-loading)4 was selected to detect the amount of drug release in different glucose concentrations. Fig. 5B shows that nanoparticles of (Insulin-loading) 4 have obvious drug release effects in different glucose concentrations. Rapid release occurred in the initial hour and then the drug release was stable and slow over the next 24 h reaching a plateau after 36 h when the glucose concentration was 3 mg/mL. The conformational changes of insulin were evaluated by CD spectroscopy. For insulin, the band at 208 nm is an alpha-helix structure, while the band at 223 nm is the beta-structure. The ratio of $[F]_{208}/[F]_{223}$ is a method of evaluating the conformational structure of insulin. In our study, it was found that the $[F]_{208}/[F]_{223}$ for standard insulin and released insulin was 1.23 and 1.15, respectively indicating that there were no significant conformational changes after release of the insulin from the nanoparticles. The results can be seen in Fig. 6.

Table 2. Insulin loading capacity and encapsulation efficiency of p(NVCL-co-AAPBA)3 NPs

Sample	Group	Insulin concentration(mg/mL)	LC(%)	EF(%)
p(NVCL -co-AAPBA)3	(Insulin-loading) 1	0.25	11.8±3.6	84.2±7.1
	(Insulin-loading) 2	0.5	12.4±3.1	75.1±10.2
	(Insulin-loading) 3	1.0	13.6±2.5	70.6±9.4
	(Insulin-loading) 4	1.5	14.2±3.1	65.3±9.1
	(Insulin-loading) 5	2.0	16.3±2.7	60.2±8.1

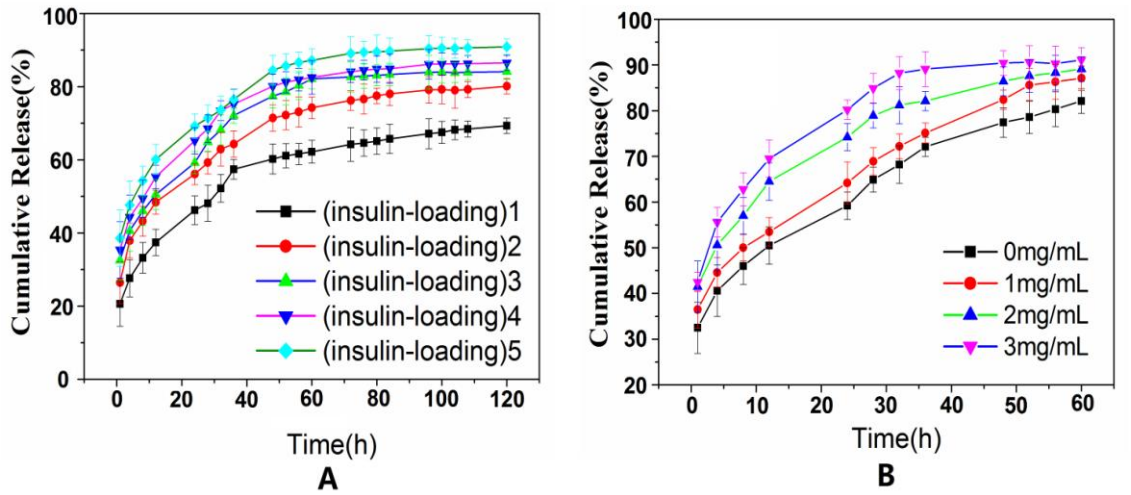

Fig. 5 *In vitro* cumulative release of insulin at pH 7.4 in PBS from: (A) different insulin-loadings on p(NVCL-co-AAPBA)3 in only PBS and (B) (Insulin-loading)4 NPs at various glucose concentrations (0, 1, 2 and 3 mg/mL).

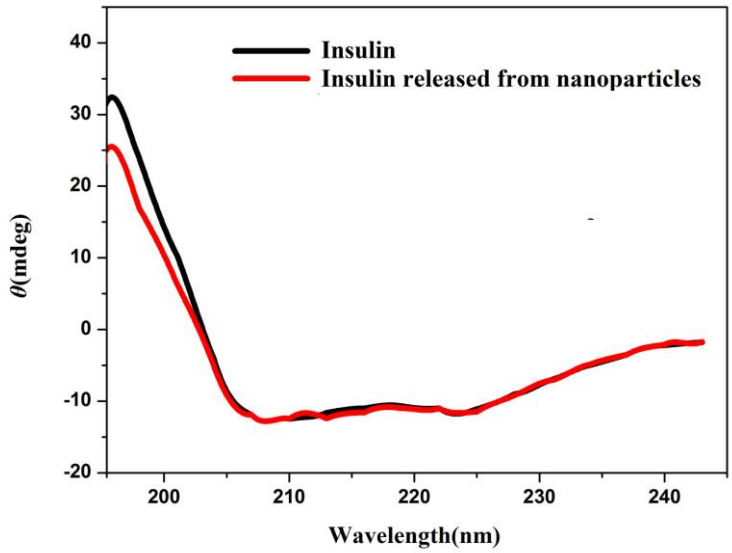

Figure 6. CD spectra of the insulin released from nanoparticles and standard insulin in PBS at pH 7.4, 25 °C.

3.5 Cytotoxicity testing

The cytotoxicity of the nanoparticles loaded with insulin and their effects on multiple cell lines were also determined. Fig. 7 shows the cytotoxicity of p(NVCL-co-AAPBA) NPs on Human hepatoma SMMC-7721 cells determined by the MTT method. The cells were exposed to suspensions of the NPs from 25 to 500 mg/mL and cells without pretreatment were used as a negative control group. After incubating for 24 h, the cell viability of glycopolymer -treated wells was higher than 100% when compared to the control group (100%). This is consistent

with the work of the Chaoxing Li group [44], indicating that the presence of the glycopolymer did not negatively impact on the cells although viability was highest with the lowest AAPBA content in the glycopolymer. Overall, these results suggest that the carbohydrate moieties in the polymers reduced the cytotoxicity and enhanced the glucose-sensitivity of the polymers bearing phenylboronic acid. Therefore, these nanoparticles have weak cytotoxicity and good cytocompatibility and thus have a potential application for *in vivo* insulin delivery.

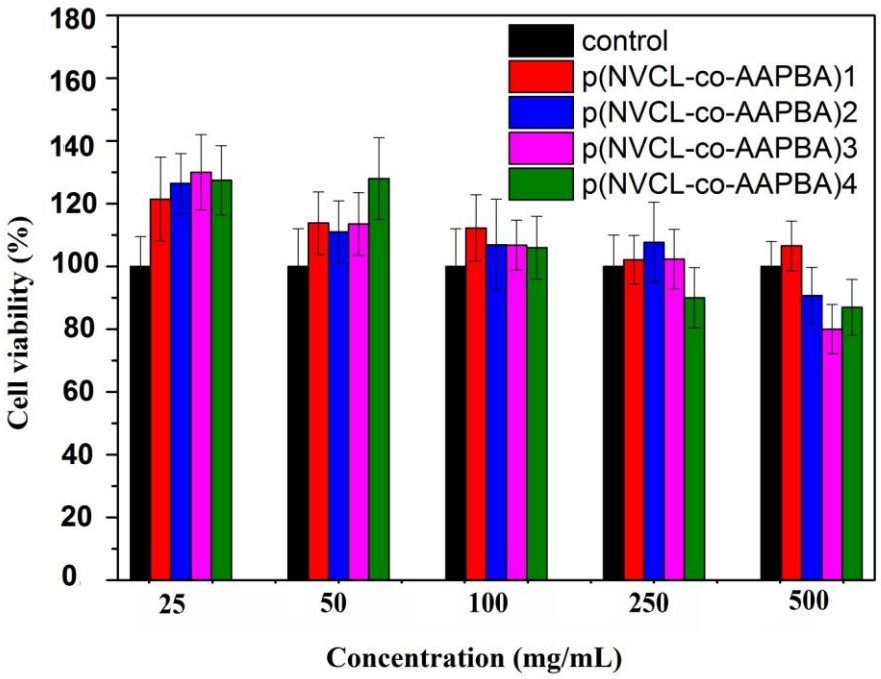

Fig. 7 Cell viability as a function of the concentration of p(NVCL-co-AAPBA) NPs by the MTT assay at 37 ^{o}C after the incubation for 24 h. Each value represents the mean ± SD (n=5)

3.6 Animal toxicity

Cytotoxicity testing cannot reflect the overall toxicity of the materials accurately since animal metabolism is not taken into account [45] so it was necessary to conduct animal toxicity tests to determine whether the materials are safe *in vivo* and the results are reported in Fig. 8 and Table 3.

Acute toxicity studies were performed by intraperitoneally injecting rats with 100, 200 and 400 mg/kg/d of NPs according to the weight of rats. During observation over 7 days it was seen that all the rats had silky hair, a good mental state and normal eating and drinking habits. After 7 days, the rats were killed and the HE staining images of their livers, kidneys, hearts, spleens and lungs were observed. As shown in Fig. 7 the organs had no obvious damage

demonstrating that the prepared materials had no toxicity *in vivo* over a short term.

Chronic toxicity was then tested with the rats being intraperitoneally injected with 10, 20, 40 and 80 mg/kg/d of NPs. The results show that the materials are safe and do not have negative impact on the blood biochemical values indicating that the materials have no toxicity over 60 days.

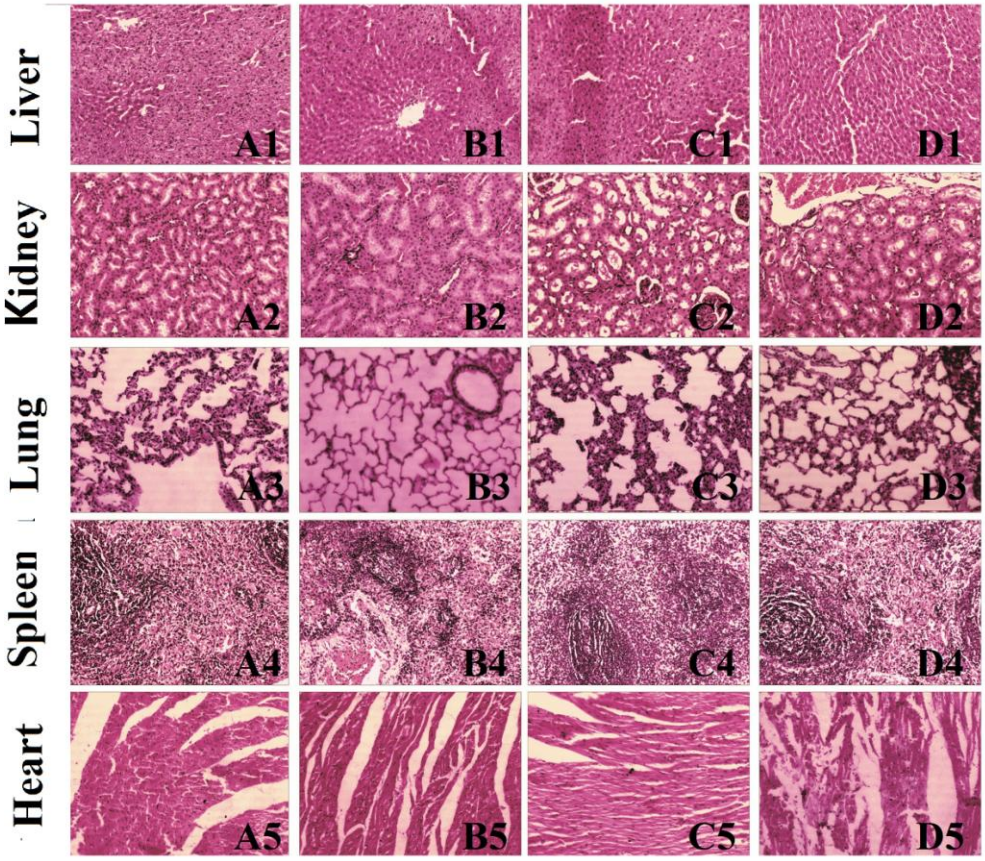

Fig. 8 Representative images of HE staining results from (A) the control group; (B) the observation group (100mg); (C) the observation group (200mg); (D) the observation group (400mg) on (1) liver; (2) kidney; (3) lung; (4) spleen; (5) heart

Table 3 Effect of administration by injection of the NPs on the biochemical parameters of rats after 70 d (n=5, mean±SD)

parameter	Control Group	Observation group(mg/kg)			
		10	20	40	80
RBC($\times10^6$/uL)	2.88±0.33	2.90±0.14	2.91±0.23	2.91±0.30	2.93±0.33
WBC($\times10^3$/uL)	9.42±0.43	9.77±0.51	9.68±0.53	9.89±0.24	10.01±0.76

Haemoglobin(g/dl)	14.44±1.21	15.12±1.65	14.98±1.23	14.92±1.16	14.85±1.44
Haematocrit(vol.%)	36.57±1.20	36.89±1.32	36.70±1.45	37.01±1.45	38.21±1.32
Platelet($\times10^3$/uL))	88.77±2.65	90.23±2.14	91.25±2.35	89.32±2.56	91.45±3.21
Serum protein (g/l)	55.89±3.45	54.36±3.54	56.44±2.79	57.21±2.91	56.62±3.74
Serum creatinine (mg/dl)	3.52±0.21	3.54±0.17	3.60±0.24	3.62±0.31	3.65±0.32
Serum glutathione (mg/dl)	0.63±0.09	0.63±0.12	0.62±0.14	0.60±0.19	0.59±0.21
Total cholesterol (mg/dl)	71.89±4.51	72.02±5.01	71.92±4.78	71.52±4.98	72.13±5.35
Glucose (mg/dl)	258.33±9.23	257.65±9.14	255.86±8.23	254.61±7.65	250.18±9.12
Uric acid (mg/dl)	5.25±0.65	5.21±0.54	5.33±0.62	5.28±0.57	5.56±0.75
AST(U/l)	39.40±3.28	40.12±3.45	39.83±2.54	40.08±3.54	40.76±4.15
ALT(U/l)	96.13±4.63	95.87±4.65	96.57±4.14	97.06±5.15	98.76±5.23

3.7 In vivo hypoglycemic studies

To quantify glucose levels, T2DM mice were given a sugar gavage and blood was then periodically taken from the tail vein for analysis at 0, 1, 2, 3, 4, 5, 6, 12, 24, 36, 48 and 60 h. The results (Fig. 9) show that the trends are the same for the samples taken at all time points with the glucose level rising rapidly for all groups except the negative control between 0 and 1 h after application of the sugar gavage. The insulin-loaded NPs and insulin injection-treated groups have very similar glucose levels from 0 h to 48 h but after 50 h, the former showed a slight increase in blood glucose levels. This suggests that insulin-loaded NPs have a stable hypoglycemic effect in the body within 48 hours, but after this time the hypoglycemic effect was significantly decreased, which may be related to the drug release and drug loading.

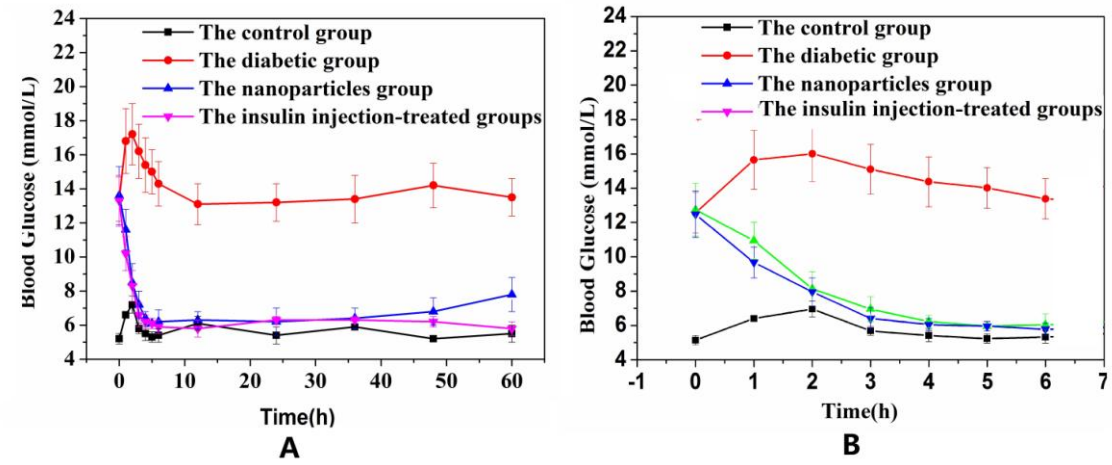

Fig. 9 Blood glucose concentration after injection: A over 60 h and B over 6 h. (Fig.8A does not show the hypoglycemic effect within first six hours, so this is presented over 6 h in Fig.8B).

4. Conclusions

Random polymers prepared from different molar ratios of AAPBA to NVCL are shown to have thermal- and glucose-sensitive properties. The random polymers could self-assemble to form NPs and TEM showed that the NPs were well dispersed as individual particles with a spherical shape. The NPs were glucose sensitive and insulin could be loaded into the NPs due to hydrophilic-hydrophobic interactions with a loading capacity of approximately 15%. Insulin release profiles revealed that there is a correlation with increasing glucose concentration. In addition, the synthetic p(NVCL-co-AAPBA) polymers are not toxic to cells and animals within the time frame of the study period probably due to the positive effect associated with the carbohydrate moieties present in NVCL which partly resolves the issue of cytotoxicity common with phenylboronic acid species. In conclusion, this research suggests that these polymers may have potential for use as future insulin delivery systems.

Acknowledgments

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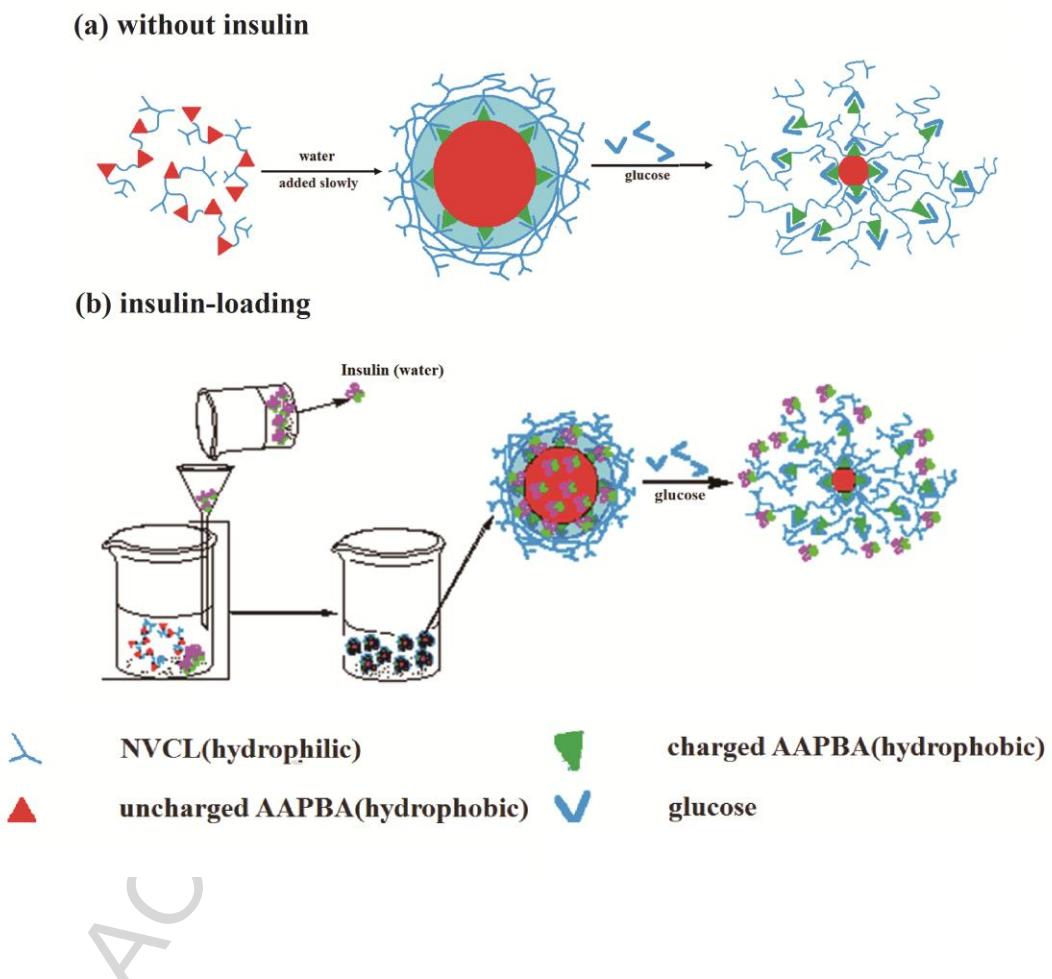
Graphical Abstract



Highlights

1. A comprehensive study of a nanoparticles may have some value for insulin or other hypoglycemic protein delivery.
2. p(NVCL-co-AAPBA)'s synthetic method is simple, convenient to carry out.
3. NVCL is low toxic and safe
4. The evaluation of acute toxicity and chronic toxicity is most highlight.